Online data supplement

Methods

Generation of Hsp20 Transgenic Mice

Hsp20 transgenic mice (FVB/N) were generated by standard procedures.[1] Animals used in this study were littermates at 12–16 weeks of age. Both males and females were used, as there were no differences between genders. All the mice were handled and maintained according to protocols approved by the Animal Care & Use Committee of the University of Cincinnati. The investigation conformed to the *Guide for the Care and Use of Laboratory Animals*, published by the National Institutes of Health.

In vivo Catheterization

To assess the effects of Hsp20 expression on left ventricular function, *in vivo* measurements of the steady-state baseline hemodynamic parameters were performed using a 1.4F micromanometer-tipped pressure volume catheter (SPR-839, Millar Instruments), as described previously.[2] Data were analyzed by Millar's PVAN software (Version 3.5)

Ex vivo Langendorff Perfusion

The effects of Hsp20 expression on left ventricular function were assessed using an isolated perfused heart model as previously described. [1] Hearts were rapidly excised and mounted on a Langendorff apparatus (~70cm H_2O perfusion pressure), perfused with Krebs-Henseleit buffer (noncirculating). A fluid-filled balloon made of plastic film was inserted into the left ventricle and attached to a pressure transducer, which was connected to a Heart Performing Analyzer (Micro-Med). A bipolar electrode (NuMed) was inserted into the right atrium, and atrial pacing was performed at 400bpm with a Grass S-5 stimulator. The continuous left ventricular pressure was measured after 30 minutes of stabilization.

Mouse Myocyte Isolation and Measurements of Mechanics and Ca- Kinetics

Isolation of mouse left ventricular myocytes was carried out as described previously.[3] Briefly, mouse hearts were excised from anesthetized (pentobarbital sodium, 70 mg/kg, i.p.) adult mice, mounted in a Langendorff perfusion apparatus, and perfused with Ca-free Tyrode solution at 37°C for 3 min. The normal Tyrode solution contained 140 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, and 5 mM HEPES, pH 7.4. Perfusion was then switched to the same solution containing 75 units/ml type 1 collagenase (Worthington), and perfusion continued until the heart became flaccid (~10–15 min). The left ventricular tissue was excised, minced, pipette-dissociated, and filtered through a 240-μm screen. The cell suspension was then sequentially washed in 25, 100, 200 μm and 1 mM Ca-Tyrode and resuspended in 1.8 mM Ca-Tyrode for further analysis. To obtain intracellular Ca signals, cells were incubated with the acetoxymethyl ester form of fura-2 (Fura-2/AM; 2 μM) for 30 min and resuspended in 1.8 mM Ca-Tyrode solution. The myocyte suspension was placed in a Plexiglas

chamber, which was positioned on the stage of an inverted epifluorescence microscope (Nikon Diaphot 200), and perfused with 1.8 mM Ca-Tyrode solution. Cell shortening and Ca-transients were measured at room temperature (22–23°C) in separate experiments, as documented below. The room temperature allowed the myocytes to be stable for up to 2 h with constant pacing. Myocytes were field stimulated to contract by a Grass S5 stimulator through platinum electrodes placed alongside the bath (0.5 Hz, bipolar pulses with voltages 50% above myocyte voltage threshold). Contractions of myocytes from random fields were videotaped and digitized on a computer. For Ca signal measurements, cells were loaded with Fura-2 (Fura-2 /AM; 2 μM) and alternately excited at 340 and 380 nm by a Delta Scan dual-beam spectrophotofluorometer (Photon Technology International) at baseline conditions and upon rapid application of 10 mM caffeine. Ca-transients were expressed as the 340/380 nm ratio of the resulting 510 nm emissions. SR Ca load was measured upon rapid application of 10 mM caffeine. Data were analyzed by Felix software (Photon Technology International).

Electrophysiology of Isolated Mouse Left Ventricular Myocytes

Isolated mouse left ventricular myocytes were maintained at room temperature (24°C) and perfused with Tyrode's solution containing (in mM) NaCl 140, KCl 5.4, $MgCl_2$ 1, $CaCl_2$ 1.8, HEPES 5, and glucose 10 (pH = 7.4). The L-type Ca- channel and the Na-Ca exchanger (NCX) currents were recorded using the whole-cell patch clamp technique with an Axonpatch-200B amplifier (Axon Instruments, Foster City, CA) , as described previously.[3, 4] NCX current was recorded using whole-cell voltage clamp at 30°C with commonly used external solution (in mM): NaCl 140, MgCl 1, Glucose 10, HEPES 5, $CaCl_2$ 2, $BaCl_2$ 1, CsCl 4, nifedipine 0.01, ryadonine 0.005 and ouabain 0.02 (pH=7.4); and internal solution (in mM): Aspartic Acid 80, CsOH 80, TEA-Cl 20, $MgCl_2$ 2.5, HEPES 10, EGTA 11, $CaCl_2$ 7.5, CsCl 15, NaCl 10 and Na_2-ATP 4 (pH=7.2). The reversal potential of NCX was calculated as $E_{(NCX)} = 3E_{(Na)} – 2E_{(Ca)}$ = 3x51–2x103 = -53 mV, which is consistent with the reversal potential observed experimentally (around -50 mV). Data collection and analysis were performed using pCLAMP software (Axon Instruments, Foster City, CA).

Quantitative Immunoblotting

Alterations in the levels of total proteins or their phosphorylation status were analyzed from whole heart homogenates or microsomal extracts by Western blotting. Briefly, an appropriate amount of heart homogenate or microsomal extract was separated by SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad). After blocking with 5 % non-fat milk, membranes were incubated with primary antibodies, followed by appropriate secondary antibodies. Primary antibodies against various proteins of interest used in this study were as follows: pSer16-PLN, pThr17-PLN and pSer2809-RyR2 (Badrilla), pSer2815-RyR2 (gift from Dr. Andrew Marks, Department of Physiology and Cellular Biophysics, Columbia University), PLN (Upstate), RyR2 (Sigma), pSer22/pSer23-TnI and TnI (Research Diagnostics Inc.), pSer282-MyBP-C (Axxora), MyBP-C (Santa Cruz), SERCA2a (custom-made commercially, Affinity

Bioreagents), LTCC (Alomone Labs), NCX (Affinity Bioreagents), CSQ (Affinity Bioreagents), PP1 (Sigma), and GAPDH (Affinity Bioreagents). The horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (1:5,000) were from Amersham Biosciences (Piscataway, NJ). The membranes were developed by an enhanced chemiluminescence Western blot analysis detection system (Amersham Biosciences). All the protein levels were quantified using AlphaEaseFC software (Alpha Innotech, San Leandro, CA).

SR Ca-Uptake

Initial SR Ca-uptake rates were determined in cardiac homogenates, using oxalate to restrict uptake to SR vesicles and $^{45}CaCl_2$, as previously described.[4] Briefly, 100-250 µg of cardiac homogenate were incubated at 37°C in a reaction buffer containing: 40 mmol/L imidazole, pH=7.0, 95 mmol/L KCl, 5 mmol/L NaN_3, 5 mmol/L $MgCl_2$, 0.5 mmol/L EGTA, and 5 mmol/L potassium oxalate. The initial uptake rates were determined over a wide range of Ca-concentrations (pCa 8 to 5). Ca-uptake into SR vesicles was initiated by addition of 5 mmol/L ATP, and aliquots were filtered through a 0.45 µm Millipore filter after 0, 30, 60 and 90 seconds. The specific ^{45}Ca-uptake values were analyzed by non-linear regression, using the OriginLab 5.1 program to obtain the Ca-affinity (EC_{50}) and the Ca-uptake rates.

Adult Rat Ventricular Myocyte Isolation and Adenovirus-Mediated Gene Transfer

Rat ventricular myocytes were isolated from adult male Sprague–Dawley rats (6–8 weeks old) and plated on laminin-coated glass coverslips or dishes, as described previously.[5] Recombinant adenoviruses Ad.GFP encoding GFP, Ad.Hsp20 containing both Hsp20 and GFP were generated by using the AdEasy-1 expression system, as described previously.[6, 7] Cardiomyocytes seeded on coverslips or dishes were infected with adenovirus in diluted media, at a multiplicity of infection (MOI) of 500, for 24 h.

Adult Mouse Cardiac Myocytes Isolation, Culture and Adenovirus-Mediated Gene Transfer

Adult mouse cardiomyocytes were isolated according to the protocols developed by the Alliance for Cellular Signaling[8] and modified by us. Briefly, adult WT and double mutant PLN mice[4] (8-10 weeks old) were anesthetized with Avertin (100 mg/kg i.p., Sigma) and hearts rapidly removed. Hearts were perfused with Krebs Henseleit Bicarbonate (KHB) buffer followed by digestion with liberase blendzyme I (0.25 mg/ml, Roche). Myocytes were dissociated by teasing the ventricles with forceps, followed by addition of 10 % serum and 12.5 µM $CaCl_2$. The concentration of $CaCl_2$ was gradually increased to 1 mM and cells were suspended in plating medium containing 10 mM of 3-butanedione monoxime (BDM). Myocytes were plated on laminin-coated (10 µg/mL) dishes for 2 hour at 5% CO_2 and 95% air at 37°C. After 2 hour of attachment, cardiac myocytes were infected with adenoviruses containing the cDNA sequence of wild type Hsp 20 (Ad.Hsp20), or green fluorescent protein (Ad.GFP) at a multiplicity of infection

(MOI) of 500 in 1 ml of 10 μM blebbistatin (Toronto research Chemicals, Canada) culture media. Experiments were performed 24 hours after infection.

Isolation of Cardiac Microsomes Enriched in SR Membranes

Briefly, hearts were homogenized at 4°C in Buffer A composed of imidazole (10 mM, pH 7.0), sucrose (300 mM), dithiothreitol (1 mM), sodium metabisulphite (1 mM), and phenylmethylsulfonyl fluoride (0.3 mM). Isolation of microsomal fractions enriched in SR membranes was achieved by differential centrifugation of the cardiac homogenate. Homogenates were centrifuged at 8,000g (20 min) and the pellets rehomogenized in buffer A and centrifuged as above. The supernatants from the two spins were combined, 4 M NaCl was added to a final concentration of 0.6 M, and centrifuged at 100,000g (60 min). The supernatant after 100,000g centrifugation was used as the cytosolic fraction. The resulting pellet was washed in Buffer A and recentrifuged at 100,000g (60 min). The final pellet was resuspended in Buffer A. The yield of protein in the final pellet was similar between wild type and transgenic hearts.

PP1 Activity Assay

Protein phosphatase activity was assessed in cardiac homogenates and cell lysates (1 μg total protein) with the Protein Serine/Threonine Phosphatase Assay System (New England Biolabs) according to the manufacturer's instructions and as described previously.[9] Okadaic acid (10 nM) was used to discern between PP1 and protein phosphatase-2A (PP2A) activities.[9]

Co-Immunoprecipitations and Immunofluorescence Studies

Association of Hsp20 with PP1 was studied by co-immunoprecipitation, as previously described. [10] A homemade anti-Hsp20 antibody[10] and anti-PP1 antibody (Sigma) were used. The immunofluorescence studies of isolated cardiomyocytes were carried out as previously described.[7] Homemade anti-Hsp20, anti-PLN (Sigma), and anti-PP1β (Thermo Scientific) antibodies were used for staining, followed by appropriate secondary antibodies (Alexa Fluor anti-rabbit 594 or Alexa Fluor anti-mouse 488, Invitrogen) with 1:500 (vol/vol) dilution in blocking buffer. Immunofluorescence images were generated from serial sections of isolated cardiomyocytes using Zeiss confocal microscopy.

Generation of Recombinant Proteins and Blot-Overlay Assay

To evaluate the interaction between PP1 and Hsp20, full length and deletion constructs of PP1 and Hsp20 were generated by PCR amplification and were subcloned in the EcoRI/SalI sites of the pMAL-c2x vector (New England Biolabs) or pGEX5x-1 vector (Amersham Biosciences), respectively. PCR was performed using primers 5' ATGTCCGACAGCGAGAAGCT 3' and 5' CATGGCAGCATGATTTCTGT 3' for full length MBP-$PP1_{(aa1-330)}$ construct, primers 5' ATGTCCGACAGCGAGAAGCT 3' and 5' CCGAATCTGCTCCATAGAC 3' for MBP-$PP1_{(aa1-187)}$, and primers 5' GCCATAGTGGACGAAAAGA 3' and 5' CATGGCAGCATGATTTCTGT 3' for MBP-$PP1_{(aa163-330)}$. Full length and deletion constructs of Hsp20 were generated by

PCR amplification, using primers 5' AGCAGGATGGAGATCCCTGT 3' and 5' CCAGCCCCCTCCTACTTG 3' for GST-Hsp20$_{(aa1-160)}$, primers 5' AGCAGGATGGAGATCCCTGT 3' and 5' GTGCTTCACGTCTAGCAGCAC 3' for GST-Hsp20$_{(aa1-82)}$ and primers 5' CACTTTTCGGTGCTGCTAGA 3' and 5' CCAGCCCCCTCCTACTTG 3' for GST-Hsp20$_{(aa73-160)}$. The sequence of all generated constructs was verified by sequencing analysis (Macrogen, Seoul, Korea). Expression of GST and MBP fusion proteins was performed, as previously described[11] and recombinant proteins were purified by affinity chromatography on Glutathione Sepharose 4B^{TM} beads (Amersham Biosciences) or amylose resin beads (New England Biolabs), according to manufacturer's instructions.

The blot-overlay assays were performed as previously described. [11] Briefly, ~2.5 µg of affinity-purified MBP, MBP-PP1$_{(aa1-330)}$, MBP-PP1$_{(aa1-187)}$, and MBP-PP1$_{(aa163-330)}$ recombinant proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Nonspecific sites on the membrane were blocked by incubation in buffer A (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% (v/v) Tween-20, 2 mM dithiothreitol, 0.5% NP-40 and 5% nonfat milk) for 16 h at 4°C. The membrane was then incubated with 3 µg/ml GST-Hsp20$_{(aa1-160)}$ fusion protein in buffer A, in the presence of 1 mM ATP, for 5 h at 25° C. Following five washes in buffer C (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% (v/v) Tween-20, 2mM dithiothreitol, 1% NP-40), the membrane was probed with anti-GST antibody (Amersham Biosciences) and bands were visualized using ECL reagents. In a set of parallel assays, 3 µg of affinity-purified GST, GST-Hsp20$_{(aa1-160)}$, GST-Hsp20$_{(aa1-82)}$ and GST-Hsp20$_{(aa73-160)}$ fusion proteins were subjected to 10% SDS-PAGE, transferred to nitrocellulose membranes, allowed to interact with MBP-PP1$_{(aa1-330)}$ protein as described above and the membrane was probed with anti-MBP antibody (New England Biolabs).

Statistics

All results are expressed as mean ± SEM. Comparisons were evaluated by Student's *t test* for two groups and one-way ANOVA for multiple groups. In all analyses, $P< 0.05$ was considered statistically significant.

References:

1 Fan G, Ren X, Qian J, Yuan Q, Nicolaou P, Wang Y, Jones W, Chu G, Kranias E. Novel cardioprotective role of a small heat-shock protein, Hsp20, against ischemia/reperfusion injury. *Circulation.* 2005;111:1792-1799.

2 Pathak A, del Monte F, Zhao W, Schultz J, Lorenz J, Bodi I, Weiser D, Hahn H, Carr A, Syed F, Mavila N, Jha L, Qian J, Marreez Y, Chen G, McGraw D, Heist E, Guerrero J, DePaoli-Roach A, Hajjar R, Kranias E. Enhancement of cardiac function and suppression of heart failure progression by inhibition of protein phosphatase 1. *Circ Res.* 2005;96:756-766.

3 Yuan Q, Fan G, Dong M, Altschafl B, Diwan A, Ren X, Hahn H, Zhao W, Waggoner J, Jones L, Jones W, Bers D, Dorn Gn, Wang H, Valdivia H, Chu G, EG K.

Sarcoplasmic reticulum calcium overloading in junctin deficiency enhances cardiac contractility but increases ventricular automaticity. *Circulation.* 2007;115:300-309.

4 Brittsan A, Ginsburg K, Chu G, Yatani A, Wolska B, Schmidt A, Asahi M, MacLennan D, Bers D, Kranias E. Chronic SR Ca2+-ATPase inhibition causes adaptive changes in cellular Ca2+ transport. *Circ Res.* 2003;92:769-776.

5 Wang X, Zingarelli B, O'Connor M, Zhang P, Adeyemo A, Kranias E, Wang Y, Fan G. Overexpression of Hsp20 prevents endotoxin-induced myocardial dysfunction and apoptosis via inhibition of NF-kappaB activation. *J Mol Cell Cardiol.* 2009;47:382 -390.

6 Fan G, Yuan Q, Song G, Wang Y, Chen G, Qian J, Zhou X, Lee Y, Ashraf M, Kranias E. Small heat-shock protein Hsp20 attenuates beta-agonist-mediated cardiac remodeling through apoptosis signal-regulating kinase 1. *Circ Res.* 2006;99:1233-1242.

7 Fan G, Chu G, Mitton B, Song Q, Yuan Q, Kranias E. Small heat-shock protein Hsp20 phosphorylation inhibits beta-agonist-induced cardiac apoptosis. *Circ Res.* 2004;94:1474-1482.

8 Sambrano G, Fraser I, Han H, Ni Y, Connell T, Yan Z, Stull J. Navigating the signalling network in mouse cardiac myocytes. *Nature.* 2002;420:712-714.

9 Rodriguez P, Mitton B, Nicolaou P, Chen G, Kranias E. Phosphorylation of human inhibitor-1 at Ser67 and/or Thr75 attenuates stimulatory effects of protein kinase A signaling in cardiac myocytes. *Am J Physiol Heart Circ Physiol.* 2007;293: H762-769.

10 Qian J, Ren X, Wang X, Zhang P, Jones WK, Molkentin JD, Fan GC, Kranias EG. Blockade of Hsp20 phosphorylation exacerbates cardiac ischemia/reperfusion injury by suppressed autophagy and increased cell death. *Circ Res.* 2009;105:1223- 1231.

11 Arvanitis D, Vafiadaki E, Fan G, Mitton B, Gregory K, Del Monte F, Kontrogianni-Konstantopoulos A, Sanoudou D, Kranias E. Histidine-rich Ca-binding protein interacts with sarcoplasmic reticulum Ca-ATPase. *Am J Physiol Heart Circ Physiol.* 2007;293:H1581-1589.

Online Figure I

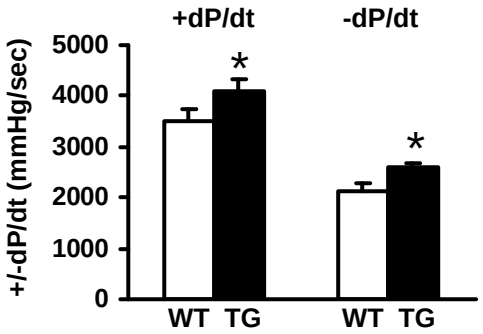

Figure. I. The left ventricular function is enhanced in Hsp20-TG heart, as measured by *ex vivo* langendorf preparations. *, $p<0.05$ vs. WT, n=5.

Online Figure II

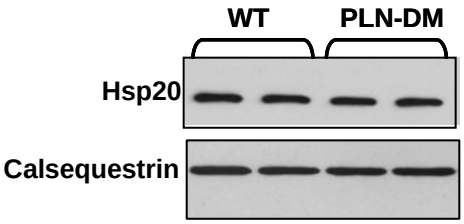

Figure II. Hsp20 expression levels was measured in the WT and PLN-DM hearts. Calsequestrin was used as a loading control.

Online Figure III

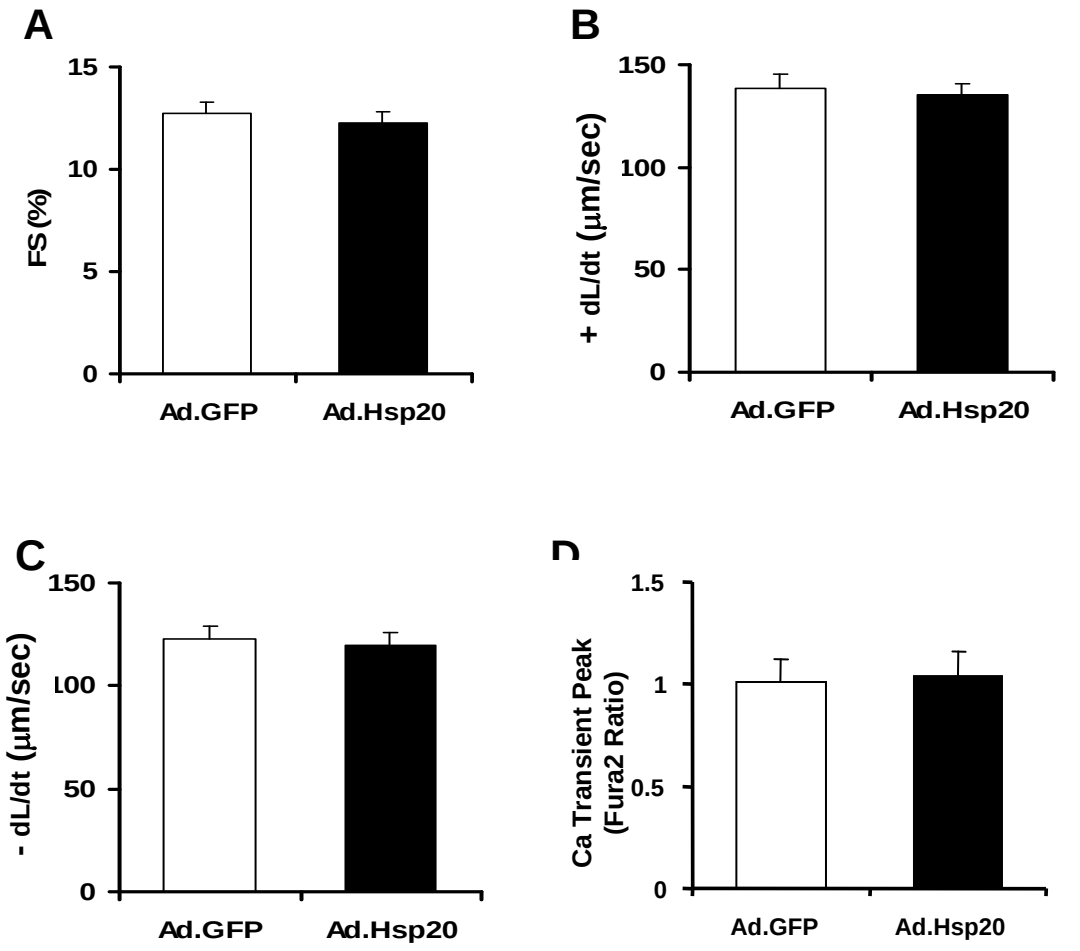

Figure III. The inotropic effects of Hsp20 are abrogated in the absence of PLN. Overexpressed Hsp20 in PLN-KO cardiomyocytes had no effects on contractility: (A) fractional shortening (FS%), (B) Maximum rates of contraction (+dL/dt), (C) maximum rates of relaxation (-dL/dt), and (D) twitch Ca amplitude in cultured WT and PLN-KO (10-12 cardiomyocytes/heart, n=3 hearts).

Online Figure IV

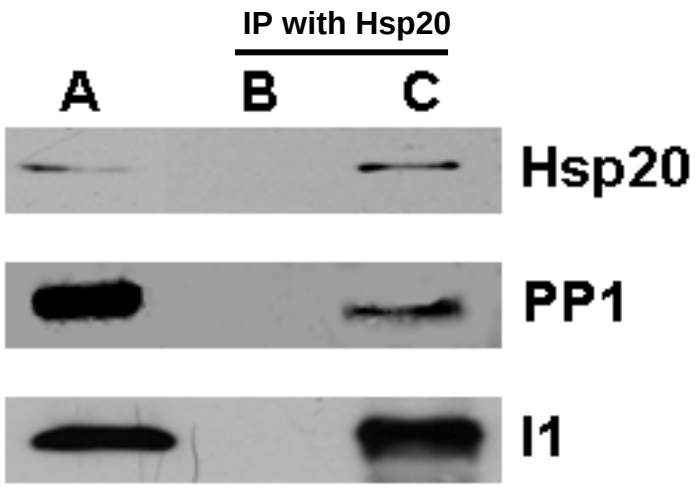

Figure IV: Immunoprecipitations (IP) using WT cardiac homogenates and the anti-Hsp20 antibody (Lane C). Lane A: Pre-IP cardiac homogenate (positive control); Lane B: agarose beads (negative control).

Online Figure V

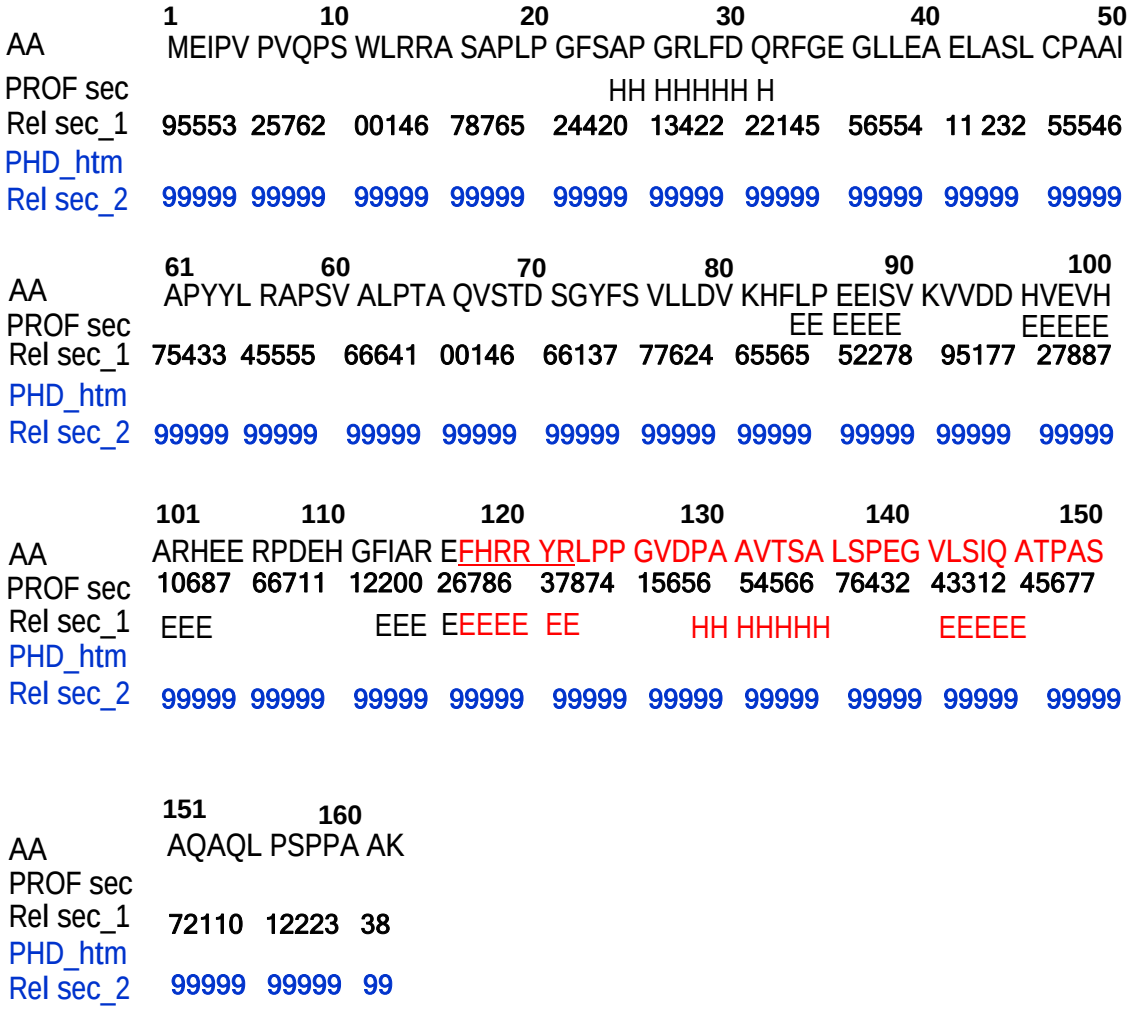

Figure V. PP1 binding motif and structure prediction of Hsp20. A putative PP1-binding motif, F-x-x-[RK]-x-[RK], has been identified in the 117 – 122 residues (underlined segment) of mouse Hsp20 amino acid sequence. The secondary structure of Hsp20 was predicted by PROF (divergent profile-based neural network prediction method, *B Rost, P Fariselli & R Casadio Prot. Science, 1996;7:1704-1718*) and PHD (simple profile-based neural network prediction method, *B Rost. Methods in Enzymology, 1996;266:525-539*). A predicted protein-protein interaction site was identified on Hsp20 sequence (residue 117-150 in red color), which overlapped the PP1-binding motif in residues 117-122. Based on PHD, there was no identified helical trans-membrane region. PROF_sec: PROF predicted secondary structure, H=helix, E=extended (sheet), blank=other (loop). Rel_sec_1: reliability index for PROF_sec prediction (0=low to 9=high). PHD predicted membrane helix: blank=non-membrane. Rel_sec_2: reliability index for PHD prediction (0=low to 9=high).